Submission under 37 C.F.R. §1.114
Application No. 10/511,725
Attorney Docket No. 042872

REMARKS

The RCE requests a suspension of action for a period of three months. Claims 1-27 are currently pending.

I. Statement of Substance of Interview

Applicants wish to thank Examiner Clark for the helpful and courteous interview conducted on June 14, 2007. The "Interview Summary" form mailed June 27, 2007 accurately memorialized the general discussion of the interview. As mentioned in the Examiner's Interview Summary, the Examiner recommended that Applicants file a RCE to obtain entry as the Examiner stated that some of the proposed changes to the claims would be considered to raise "new issues" after final. Some specific points discussed during the interview are also discussed below.

It is respectfully submitted that the instant Statement of Substance of Interview complies with the requirements of 37 C.F.R. §§1.2 and 1.133 and MPEP §713.04.

II. Clarification to Page 26 of the Specification

Page 26, last line, is amended to change "documents 2 to 4" to "Documents 1 to 3". "Documents 2-4" or page 26 was a mistake for "Documents 1-3." Documents 1-3 of this application were "Documents 1-3" in the Japanese Priority Application 2002-250991. However, in the Japanese Priority Application 2002-250992, while the documents cited are the same, the order of the listed documents was changed. The instant U.S. application listed order of the documents the same as Japanese Priority Application 2002-250991 on pages 3-6 of Applicants'

specification. Therefore, the text of the application should also follow the listed order of the documents the same as Japanese Priority Application 2002-250991.

See also the instant U.S. application, the paragraph bridging pages 3-4, the first full paragraph on page, the first full paragraph on page 13, and the first full paragraph on page 40 for support for the change.

JP Application 2002-250991

Document 1 Reference "Nihon Eiyou · Shokuryou Gakkai Soukai Kouen Youshishu (Japan Nuturition · Executive Summary of Dietary Academic Conference Lectures)," Vol. 53, 53 (1999);

Document 2 Reference "Nihon Jyouzou Kyoukai Shi (Japan Brewing Association Journal)," Vol. 94, No. 9, 768 (1999);

Document 3 Reference "Nihon Jyouzou Kyoukai Shi (Japan Brewing Association Journal)," Vol. 95, No. 9, 706 (2000);

Document 4 Japanese Unexamined Patent Publication 2001-145472; and

Document 5 Japanese Unexamined Patent Publication 1994-98750

JP Application 2002-250992

Document 1 Japanese Published Patent 1994-98750 bulletin;

Document 2 Reference "Nihon Eiyou · Shokuryou Gakkai Soukai Kouen Youshishu (Japan Nuturition · Executive Summary of Dietary Academic Conference Lectures)," Vol. 53, 53 (1999);

Document 3 Reference "Nihon Jyouzou Kyoukai Shi (Japan Brewing Association Journal)," Vol. 94, No. 9, 768 (1999);

Document 4 Reference "Nihon Jyouzou Kyoukai Shi (Japan Brewing Association Journal)," Vol. 95, No. 9, 706 (2000); and

Document 5 Japanese Unexamined Patent Publication 2001-145472.

III. The Rejection under 35 U.S.C. §112

Claims 1-7 are rejected under 35 U.S.C. §112, second paragraph, as allegedly being indefinite.

Claims 1, 2, 4 and 6 and claims 3, 5 and 7 remain rejected under 35 U.S.C. §112, second paragraph, as allegedly being indefinite for the same reasons as set forth in the first Office Action.

The Examiner states that the terms "a barley" in claim 1, line 6, "the amino acids" in claim 1, lines 14 and 15, and "said compound" in claim 1, line 16 have antecedent basis problems.

The Examiner still considers the term "barley" to be indefinite. The Examiner states that Applicant may overcome the rejection by placing the genus-species name of "barley" in parentheses after the term "barley".

Claim 1 has been amended for clarity to correct the antecedent basis problems. Applicants note that the first use of the term "barley" is in connection with a "barly sochu stillage." Applicants have amended claim 1 to recite *Hordeum vulgar* L., a Latin "genus-species" name in parenthesis after the term "barley". Applicants note that the term barley refers to a plant species known as *Hordeum vulgar* L. and that said term includes various forms, including

various rows based on the number of kernel rows and hulless, etc.

For the above reasons, it is respectfully submitted that Applicants' claims are clear and definite and it is requested that the rejection under 35 U.S.C. §112 be reconsidered and withdrawn.

IV. The Rejection under 35 U.S.C. §112

Claims 1-7 are rejected under 35 U.S.C. §112, first paragraph, as alleged failing to comply with the enablement requirement.

During the interview, support in the specification for treating types of "alcoholic hepatopathy" was discussed.

Further to the Examiner's position concerning the conclusions to be found based on testing on rats, submitted herewith are three journal articles, Baraona (Fatty Liver, Hyperlipemia, and Erythrocyte Alterations Produced by Ethanol Feeding in the Rat), Lieber (Alcohol, Nutrition, and the Liver) and Lieber et al (Sequential Production of Fatty Liver, Hepatitis, and Cirrhosis in Sub-Human Primates Fed Ethanol with Adequate Diets). The three journal articles discuss the use of rats as a tool in evaluating alcohol related disease.

Applicants have amended claim 1 to remove the recitation concerning inhibiting the onset of alcoholic hepatopathy and/or capable of healing alcoholic hepatopathy. However, new claim 27 has been added which includes the language "the composition is capable of treating the onset of alcoholic hepatopathy in a patient in need thereof." Applicants respectfully submit that the present specification fully supports amended claim 1 and new claim 27.

Also, during the interview, the distillation process in general, the meaning of the term "stillage" and the meaning of "Shochu" was also discussed. Attached is a definition of the term "stillage" as the grains and liquid effluent remaining after distillation. (http://cancerweb.ncl.ac.uk/cgi-bin/omd?stillage attached.) As to the Examiner's position concerning enablement and the synthetic absorbent, Applicants respectfully submit that one skilled in the art could make and use Applicants' claimed invention without undue experimentation.

Applicants submit that their disclosure is fully enabling and request that the rejection under 35 U.S.C. § 112, second paragraph, be reconsidered and withdrawn. It is respectfully submitted that one of ordinary skill in the art would be able to practice Applicants' invention without undue experimentation.

V. **The Rejection Based on Omori et al**

Claims 1-4 are rejected under 35 U.S.C. §102(a) as being anticipated by Omori et al.

Claims 1-7 are under 35 U.S.C. §103(a) as allegedly being unpatentable over Omori et al in view of Kaneuchi et al.

Applicants respectfully submit that the present invention is not anticipated by or obvious over the disclosures of Omori et al, either alone or in combination with Kaneuchi et al, and request that the Examiner reconsider and withdraw this rejection in view of the following remarks.

Claim 1 has been amended to recite the presence of free saccharides and polysaccharides, wherein the free saccharides have a saccharide composition comprising from 2 to 6% by weight

of glucose, from 0.5 to 5% by weight of xylose and from 0.5 to 3% by weight of arabinose, and the polysaccharides have a saccharide composition comprising from 6 to 16% by weight of glucose, from 3 to 12% by weight of xylose and from 0.5 to 4% by weight of arabinose. Applicants respectfully submit that the total amount of polysaccharides and the proportion and the content of xylose which comprises the polysaccharides differ from those of Omori et al.

Regarding the process steps of making the claimed composition, the present invention uses a synthetic absorbent process conducted after a solid-liquid separation. By condensing comparatively short chained peptides with an average chain length of 3-5, amino acids, and polysaccharides, etc. in the residual liquid of distilled barley shochu, as well as with an aim to obtain the composition as noted in the claim.

On the other hand, Omori discloses a sedimentation process conducted with ethanol, by conducting a process step of solubilization of insoluble hemicellulose which exists in the residual liquid of distilled barley shochu using alkaline, with an aim of condensing/extracting polysaccharides with comparatively large molecular weight (especially hemicellulose) and protein.

In Omori, the residual liquid of distilled shochu is a starting composition, and therefore, with the effect of fermentation of aspergillus and yeast etc. in the process step of shochu, to some extent, the molecular weight of the composition is made into a low molecular weight, but within that, when alcohol is added, the aim is to collect comparatively large molecular weight components that settles.

Therefore, the present invention and the disclosures of Omori are different in terms of their aim and the method and composition obtained. The composition that results form the claimed process and from the different process of Omori are different. First, as previously mentioned, the amount of organic acid is clearly different between the two. Further, the total amount of polysaccharides and the proportion and the content of xylose which comprises the polysaccharides differ. See the discussion of Document 4 (Omori et al) in Applicants' specification, for example at page 12.

For the above reasons, it is respectfully submitted that the subject matter of claims 1-7 and 27 is neither taught by nor made obvious from the disclosures of Omori et al, either alone or in combination with Kaneuchi et al, and it is requested that the rejections under 35 U.S.C. §§102(a) and 103(a) be reconsidered and withdrawn.

VI. Conclusion

In view of the above, Applicants respectfully submit that their claimed invention is allowable and ask that the rejections under 35 U.S.C. §112 and the rejections under 35 U.S.C. §§102 and 103 be reconsidered and withdrawn. Applicants respectfully submit that this case is in condition for allowance and allowance is respectfully solicited.

If any points remain at issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the local exchange number listed below.

Submission under 37 C.F.R. §1.114
Application No. 10/511,725
Attorney Docket No. 042872

If this paper is not timely filed, Applicants respectfully petition for an appropriate extension of time. The fees for such an extension or any other fees that may be due with respect to this paper may be charged to Deposit Account No. 50-2866.

Respectfully submitted,

WESTERMAN, HATTORI, DANIELS & ADRIAN, LLP

Lee C. Wright
Attorney for Applicants
Registration No. 41,441
Telephone: (202) 822-1100
Facsimile: (202) 822-1111

LCW/af

I hereby certify that the attached Applicants hereby transmit the attached Amendment under 37 C.F.R. §1.114 (19p) are being **formally transmitted** via the USPTO Central Fax No.571-273-8300 on **July 2, 2007**.

Lee C. Wright
Reg. No. 41,441

THE AMERICAN JOURNAL OF CLINICAL NUTRITION
Vol. 22, No. 3, March, 1969, pp. 356–357
Printed in U.S.A.

Fatty Liver, Hyperlipemia, and Erythrocyte Alterations Produced by Ethanol Feeding in the Rat[1,2]

ENRIQUE BARAONA AND CHARLES S. LIEBER

THE ASSOCIATION OF HYPERLIPEMIA, fatty liver, and hemolytic anemia has been described in alcoholic patients (Zieve's syndrome) (1). The present study was aimed at reproducing this syndrome experimentally. For 24 days rats were pair-fed liquid diets containing 36% of total calories either as ethanol or as carbohydrate (controls) (2). It was confirmed that under these conditions, ethanol ingestion resulted in fatty liver production. To simulate meal-feeding and to control rates of food intake, the diets were administered by gastric tube 18 hr, 6 hr, and 90 min before the rats were killed. Alcohol produced an increase in the concentration of all serum lipoprotein fractions with a predominant effect in the very low density fraction ($d < 1.019$) in which the lipid content doubled. In addition, the plasma hemoglobin (measured as cyanmethemoglobin in the clear infranates after separation of lipoproteins by ultracentrifugation) increased significantly from 0.29 ± 0.07 mg/ml of plasma in the controls to 0.67 ± 0.10 mg/ml ($P < 0.01$) in the alcohol-treated animals. There was a good correlation ($r = 0.825$) between the amount of hemoglobin in the plasma and the degree of hyperlipemia. Hematocrit values were unaffected by ethanol, but the percentage of reticulocytes increased slightly from $0.60 \pm 0.09\%$ in the controls to $1.17 \pm 0.13\%$ after ethanol ($P < 0.05$). This suggests that the hemoglobinemia may result from intravascular hemolysis.

In a second group of animals the period of gastric-tube feeding was extended to 78 hr. Similar blood lipid, hemoglobin, and reticulocyte changes were produced by ethanol. In addition, the osmotic fragility of the erythrocytes was measured (corrected to pH 7.4) and found to be slightly but significantly altered ($P < 0.01$): 50% hemolysis occurred at a NaCl concentration of 0.498 ± 0.006 g/100 ml in the controls and 0.512 ± 0.005 in the alcohol-fed animals.

In view of the known rapid exchange of lipids between red cells and plasma (3, 4) and of the effects of other dietary-induced hyperlipemias on red cell survival (5, 6), the serum lipid changes produced by alcohol could be causally related to the appearance of the hemoglobin in the plasma and to the other red cell changes. The experimental model described here could be used to investigate the pathogenesis of the early changes of the "Zieve's syndrome."

In summary, an experimental model has been described in the rat in which alcohol

[1] From the Section of Liver Disease and Nutrition, Bronx Veterans Hospital, and the Department of Medicine, Mount Sinai School of Medicine, City University of New York, New York, New York.

[2] This work was supported by Public Health Service Research Grants MH 14263, MH 15558, AM 12511, and AM 10893, and by International Postdoctoral Research Fellowship FO 5 TW 996 (to Dr. E. Baraona).

administration results in the association of fatty liver, hyperlipemia, and evidence for mild hemolysis.

REFERENCES

1. ZIEVE, L. Jaundice, hyperlipemia and hemolytic anemia: a heretofore unrecognized syndrome associated with alcoholic fatty liver and cirrhosis. *Ann. Internal Med.* 48: 471, 1958.
2. DECARLI, L. M., AND C. S. LIEBER. Fatty liver in the rat after prolonged intake of ethanol with a nutritionally adequate new liquid diet. *J. Nutr.* 91: 331, 1967.
3. MURPHY, J. R. Erythrocyte metabolism. Equilibration of cholesterol-4-C^{14} between erythrocytes and variously treated sera. *J. Lab. Clin. Med.* 60: 571, 1962.
4. REED, C. F., M. MURPHY AND G. ROBERTS. Phospholipid exchange between plasma and erythrocytes in man and the dog. *J. Clin. Invest.* 47: 749, 1968.
5. PRIEST, R. E., AND S. J. NORMANN. Diet-induced hemolytic anemia in the rat. Concomitant renal hemosiderosis. *Arch. Pathol.* 74: 423, 1962.
6. YAMANAKA, W., H. S. WINCHELL AND R. OSTWALD. Erythrokinetics in dietary hypercholesteremia of guinea pigs. *Am. J. Physiol.* 213: 1278, 1967.

Alcohol, nutrition, and the liver[1,2]

C. S. Lieber

First of all I wish to express my gratitude to the American Society for Clinical Nutrition and to the National Dairy Council for honoring me with the McCollum Award, which I accept not only in my name but especially in the name of my past and present co-workers. They are too numerous to all be cited here but I wish to single out Miss L. M. DeCarli with whom I had the privilege of being associated for the last 15 years, as well as my other present collaborators, Drs. E. Baraona, L. Feinman, Y. Hasumura, R. C. Pirola, E. Rubin, and R. Teschke. I had the good fortune to be introduced to the relationship of nutrition to liver disease by Dr. C. S. Davidson at the Thorndike Memorial Laboratory and the Harvard Medical Services of the Boston City Hospital. There I also witnessed how Drs. W. B. Castle, M. Finland, V. Herbert, and others made medical history in nutrition and clinical investigations through the ingenious blend of bedside observation and modern technology.

My initial efforts focused on the pathogenesis of alcohol-induced diseases because a majority of my patients with liver disease are alcoholics. A large number of these are under- or malnourished and, obviously, nutritional factors play an important role in the development of alcoholic liver disease, including its first stage, the fatty liver. Because fatty livers could be produced in experimental animals that were given severely deficient diets even in the absence of alcohol, it was not unexpected that the prevailing view at the time was that liver disease in the alcoholic was solely due to nutritional deficiencies and not to alcohol itself. It was believed that alcohol was acting merely by providing calories, and that when given with an adequate diet, alcohol calories were not more toxic than other calories, such as those of lipids and carbohydrates. As a consequence, it was common practice to tell the alcoholic who refused to stop drinking that he could avoid liver disease by maintaining an adequate diet. It appeared to me, however, that this was not necessarily effective in preventing liver disease and that a new approach was needed.

First, we had to assess to what extent a conventionally adequate or even an enriched diet can prevent alcohol from affecting the liver. In view of the importance of this question, we felt justified to test this in volunteers and we soon found that the effect of alcohol on the liver could not be alleviated merely by an across-the-board nutritional supplementation. As fatty liver was one of the first manifestations of this direct effect of alcohol, we were prompted to investigate the effects of ethanol upon lipid and intermediary metabolism. To achieve this, an experimental model was required. However, with conventional feeding techniques, that is, incorporation of ethanol in drinking water, rats refuse to take amounts of alcohol sufficient to develop liver injury if the diet is adequate. Rats given alcohol in drinking water develop liver injury only if the diet is deficient in protein and lipotropic factors, an observation which contributed to the notion that alcohol has no harmful action of its own. But with this model, alcohol intake is so low that we could hardly detect any alcohol in the blood. We finally succeeded in overcoming the natural aversion of the rat to alcohol by incorporating the ethanol in a totally liquid diet. It is only fitting to acknowledge the help that the dairy industry gave me in developing this model. It was indeed from a local milk company that I learned how to stabilize our diet. The rats were given nothing

[1] Acceptance speech delivered at the McCollum Award luncheon of the American Society for Clinical Nutrition, Atlantic City, New Jersey, April 28, 1973.

[2] Most of the studies mentioned were supported in part by Public Health Service Grants AA00224 and AM12511 from the National Institutes of Health, Bethesda, Maryland 20014, and by the Veterans Administration.

but these liquid diets, and therefore in order to eat or to drink, they had no choice but to take the alcohol with it. In this way, we succeeded in achieving significant blood alcohol levels and also striking changes in the liver, whereas littermates pair-fed with isocaloric amounts of control diets continued to grow and had normal livers. Once we had this experimental model, it was merely a matter of time to answer a number of obvious questions. The first was to determine the origin of the fat that accumulates in the liver. When alcohol is given with a fat containing diet, lipids of dietary origin are deposited in the liver, whereas when alcohol is given with a low fat diet, newly synthesized lipids are found. As a corollary, a decrease in the fat content of the diet resulted in a reduction in the capacity of alcohol to produce a fatty liver. Not only is the amount of the fat in the diet important but its nature also matters; a substitution of long-chain by medium-chain triglycerides decreased the extent of the fatty liver. Using the same model, we studied the role of dietary protein; a severe deficiency potentiated the effect of alcohol both in the rat and in nonhuman primates. The converse, however, was not true. Although a deficiency in nutrients exaggerated the ethanol effect, a large supplementation of protein could not fully prevent it. Therefore, it became important to define the biochemical mechanism of this change induced by ethanol, with the hope that an understanding of the pathogenesis may eventually lead to a rational form of prevention or therapy. We wondered, of course, how alcohol causes fat deposition in the liver and a relatively simple answer evolved. When we ingest large amounts of alcohol, the body is presented with a major problem of disposal, i.e., both the kidney and lungs are inefficient in excreting alcohol. Furthermore, unlike other nutrients (such as lipids or carbohydrates), alcohol cannot be stored in the body. The only effective way to rid the body of the ethanol is oxidation and the only organ which contains a significant amount of the enzymes needed is the liver. Unfortunately, we sometimes tend to ingest large amounts of alcohol. For instance, a fifth of whiskey (760 ml) contains almost 2,000 kcal, which represents a tremendous metabolic overload for the liver. Under these conditions, alcohol becomes the preferred fuel. The liver ceases to burn its regular fuel, namely fat, and a number of consequences ensue: *1*) the fat that is not being utilized accumulates, and *2*) ethanol oxidation results in the generation of a large amount of hydrogen, part of which is incorporated into lactate, causing hyperlacticacidemia and acidosis. The hyperlacticacidemia prevents the kidney from excreting uric acid, which results in a previously unrecognized form of secondary hyperuricemia. Another way to dispose of the excess hydrogen is to make more fat. Theoretically, increased lipogenesis in the liver can be considered as one way for the liver to cope with this metabolic overload produced by alcohol. The liver then has to rid itself of the fat which it does in two ways. First, fat is converted, in part, to ketones; this, in some predisposed individuals, will result in severe ketoacidosis. Secondly, another way to dispose of the fat is to secrete lipoproteins; hyperlipemia is indeed commonly associated with alcoholism. The liver adapts to this task by proliferation of those organelles which are involved in the export of lipids, namely, the endoplasmic reticulum (also called the microsomal fraction when obtained by ultracentrifugation). A comparable proliferation of the endoplasmic reticulum is observed after administration of other drugs (such as phenobarbital), because these membranes are the site of detoxification of foreign compounds. This, we found, also applies to alcohol. Indeed, in addition to the accepted alcohol dehydrogenase (ADH) pathway, the microsomes are capable of ethanol metabolism. Unlike ADH, this microsomal ethanol oxidizing system (MEOS) has the adaptive capacity to increase in activity after alcohol feeding. This helps the body in disposing of the alcohol, but at a price: there is no coupling of oxidation to phosphorylation in the microsomes. Thus, heat is produced without conservation of chemical energy. To the extent that heat production exceeds the needs for thermoregulation, it represents energy wastage. Thus, in an alcoholic who takes large amounts of alcohol, alcohol calories may not be fully utilized, not only because sometimes there is malabsorption or maldigestion of nutrients, but also for reasons of energy waste. In any event, by increasing microsomal mass and function, chronic alcohol consumption enhances not only tolerance for alcohol but also increases our capacity to rid ourselves of other drugs, food additives, and insecticides, and, in that way,

alcohol may conceivably help us survive in modern society.

The adaptive mechanisms, however, can be overwhelmed and one of the consequences is fat accumulation. Fat by itself may not be toxic but its accumulation is a witness of the severe metabolic disturbance and the latter is probably the cause for more permanent damage; decreased mitochondrial function is followed by altered mitochondrial structure. Mitochondrial "death" is seen early in the disease; later, cell death or necrosis and alcoholic hepatitis appear. We do not know how the hepatitis develops, but we have some expectation of unraveling this in our colony of baboons fed alcohol. This latter species does indeed offer a new experimental model for the more severe stage of alcohol-induced liver injury. In any event, in man, necrosis is eventually followed by scarring, or fibrosis, or cirrhosis. Excess collagen may accumulate as a response to the necrosis and also because alcohol promotes collagen production in the liver by increasing peptidylproline hydroxylase activity. I do not know to what extent alterations in collagen metabolism play a direct role in the development of cirrhosis. I do hope, however, that if I continue to be blessed with such remarkable collaborators, and provided that our research remains funded, we may scratch deeper under the surface of the cirrhotic liver.

Thank you again for honoring my team.

Reference

1. LIEBER, C. S. Liver adaptation and injury in alcoholism. New Engl. J. Med. 288: 356, 1973.

PNAS

Sequential Production of Fatty Liver, Hepatitis, and Cirrhosis in Sub-Human Primates Fed Ethanol with Adequate Diets

Charles S. Lieber, Leonore M. DeCarli, and Emanuel Rubin

PNAS 1975;72;437-441
doi:10.1073/pnas.72.2.437

Proc. Nat. Acad. Sci. USA
Vol. 72, No. 2, pp. 437–441, February 1975

Sequential Production of Fatty Liver, Hepatitis, and Cirrhosis in Sub-Human Primates Fed Ethanol with Adequate Diets

(alcoholism/fibrosis/microsomes/mitochondria/liquid diets)

CHARLES S. LIEBER*†, LEONORE M. DECARLI*†, AND EMANUEL RUBIN‡

* Section of Liver Disease and Nutrition, Veterans Administration Hospital, Bronx, New York 10468; and † Departments of Medicine and ‡ Pathology, Mt. Sinai School of Medicine of the City University of New York, New York, N.Y. 10029

Communicated by Ludwik Gross, November 11, 1974

ABSTRACT This study reproduces in experimental animals the sequential development of all the liver lesions seen in the human alcoholic: in 15 baboons fed ethanol, all developed fatty liver, five progressed to hepatitis, and five had cirrhosis. Maintenance of a nutritionally adequate regimen despite the intake of inebriating amounts of ethanol (50% of total calories) was achieved by incorporation of the ethanol in a totally liquid diet. Upon ethanol withdrawal, signs of physical dependence, such as seizures and tremors, developed. Ultrastructural changes of the mitochondria and the endoplasmic reticulum were already present at the fatty liver stage and persisted throughout the hepatitis and cirrhosis. The lesions were similar to those observed in alcoholics (including the inflammation and the central sclerosis) and differed from the alterations produced by choline and protein deficiencies. At the fatty liver stage, some "adaptive" increases in activity of microsomal enzymes [aniline hydroxylase (EC 1.14.14.1) and the microsomal ethanol oxidizing system] were observed, but these tended to disappear with the development of hepatitis and cirrhosis. Fat accumulation was also much more pronounced in the animals with the hepatitis as compared with those with simple fatty liver (an 18-fold compared with 3- to 4-fold increase in liver triglycerides). The demonstration that these lesions can develop despite an adequate diet indicates that in addition to correction of the nutritional status, control of alcohol intake is mandatory for the management of patients with alcoholic liver injury.

With increasing alcohol consumption, the incidence of related complications has been rising steadily, particularly that of associated liver disease, to the extent that at the present time, cirrhosis of the liver, the most severe hepatic complication of alcoholism, is the third cause of all deaths between the ages of 25 and 65 in the city of New York (1). In addition to cirrhosis of the liver characterized by diffuse hepatic scarring, alcohol abuse is also associated with hepatic inflammation and necrosis (alcoholic hepatitis) and excess fat accumulation (alcoholic fatty liver). The relationship of these various liver injuries to each other, however, has been questioned. Furthermore, since not all alcoholics develop liver injury, there has been considerable debate concerning the question whether alcohol itself or some associated factor, such as dietary deficiency, is the main cause for the liver disease. The question has both theoretical and practical implications. The classic belief that liver injury could be prevented in the alcoholic by merely controlling the diet was challenged by evidence that alcohol might exert direct toxic effects upon the liver; it was indeed shown that the fatty liver, the most benign stage of the disease, could be produced in volunteers given alcohol in association with adequate or enriched diets (2–4). Fatty liver, however, is still a fully reversible lesion and the question remained whether alcoholic hepatitis, associated with a high morbidity, and irreversible cirrhosis could also be linked directly to alcohol ingestion itself rather than to a deficient diet. This problem could not be studied in volunteers, in view of the severity of the lesions involved. Previous attempts to produce these lesions in animals failed because of the reluctance of all species used to consume enough alcohol when the latter was given as part of the drinking water. This natural aversion for ethanol was overcome by the incorporation of ethanol in totally liquid diets. This new experimental model for alcohol feeding showed that even when given with adequate diets, ethanol can cause alcoholic hepatitis and cirrhosis in nonhuman primates. The development of this new experimental model clarifies the question of the etiology of liver injury associated with alcohol abuse and represents a new tool for the development of rational forms of prophylaxis and therapy.

MATERIALS AND METHODS

The detailed composition of the liquid diet fed to the baboons is given elsewhere (5). The protein content (18% of total calories) corresponds to that of commonly used commercial diets that are satisfactory for the baboon and is almost twice the amount recommended for human diets (6). The mineral and vitamin content of the diet exceeded the requirements formulated for the monkey (7–9). Its caloric value was 1 calorie/ml. The diet was prepared by Bio Serv Inc., Frenchtown, N.J., and was given to the baboons twice a day in standard drinking bottles equipped with an outlet valve. Each alcohol-fed animal was matched with a control, the dietary intake of which was identical except for the isocaloric substitution of carbohydrate by ethanol to the extent of 50% of total calories. This technique of daily pair feeding was adopted to assure a strictly equal caloric intake in both ethanol-treated animals and in their individual pair-fed controls.

The 30 adolescent or young animals used for this study were either *Papio hamadryas* or olive and yellow baboons. Twelve animals were raised in this country, whereas the remainder were imported from Africa and were studied after prolonged quarantine periods. They were housed in individual cages at the Laboratory for Experimental Medicine and Surgery in Primates (LEMSIP), Tuxedo, N.Y. Until the actual study period, they were given a routine regimen of Purina® monkey chow *ad libitum*, supplemented with a daily vitamin prep-

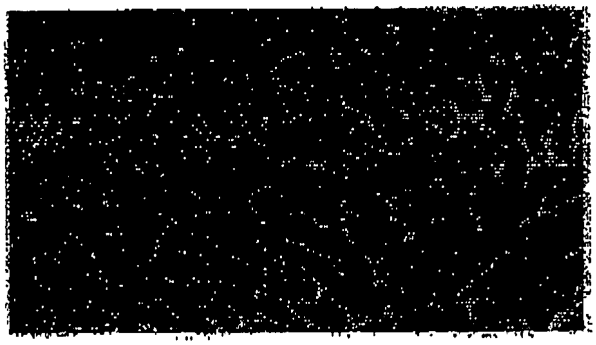

FIG. 1. Section of liver of baboon no. 536 before administration of ethanol. The architecture is normal, with the usual relation of portal tracts (PT) and central veins (CV). No fat, cell degeneration, or inflammation is present. Hematoxylin and eosin (magnification: ×111).

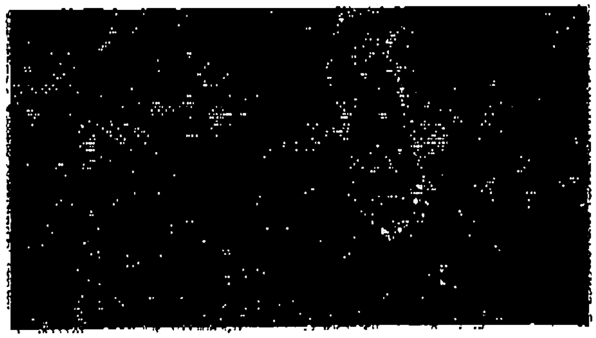

FIG. 3. High power view of liver shown in Fig. 2. Numerous ballooned hepatocytes are located around a thickened central vein (CV). Irregular eosinophilic cytoplasmic inclusions, resembling human alcoholic hyaline, are present (arrows). Hematoxylin and eosin (magnification: ×222).

aration. The animals entered the study after prolonged observation and after repeated hematological and stool examinations had indicated the absence of disease.

Surgical biopsies of the liver were performed at regular intervals under anesthesia. Samples were taken for analysis of total lipids and triglycerides, light and electron microscopy, and alcohol dehydrogenase activity, as described (10). The activity of the microsomal ethanol-oxidizing system (11) and that of aniline hydroxylase (12) were determined in liver microsomes. Blood samples were taken for the measurement of ethanol (13) and of cholesterol, albumin, bilirubin, alkaline phosphatase activity, serum glutamic-oxaloacetic transaminase (EC 2.6.1.1; L-aspartate:2-oxoglutarate aminotransferase) activity, creatinine, urea, and glucose in a Technicon Autoanalyzer. The absence of hepatitis-associated antigen in the blood was verified by radioimmunoassay.

RESULTS

Nine pairs of animals were pair-fed alcohol-containing or the control liquid diet for 8–22 months. The average duration of the treatment was 15 months, and the mean intake was 80.0 $\pm$ 2.24 ml/kg per day. The alcohol-fed baboons and their controls had average initial weights of 10.6 $\pm$ 0.35 and 10.6 $\pm$ 0.33 kg, respectively. The baboons fed alcohol maintained their weight (10.2 $\pm$ 0.46 kg) throughout the study, whereas the controls increased their weight to an average of 11.8 $\pm$ 0.34 kg ($P < 0.01$). Liver biopsies obtained prior to the start of the study revealed normal morphology (Fig. 1), and no abnormalities developed in the controls. In the animals drinking the alcohol-containing diet, inebriation was commonly observed. Blood ethanol concentrations in inebriated animals were 262 and 258 mg/100 ml on two occasions in one baboon and 358 and 376 on one occasion in two other animals. Alcohol consumption resulted in the development of a fatty liver, with an average triglyceride content of 144 $\pm$ 36 compared to 10 $\pm$ 2 mg/g in the control ($P < 0.01$). Four pairs of animals were biopsied sequentially after 8.5 and 21 months; whereas the triglyceride was only 62 $\pm$ 19 mg/g after 8.5 months of alcohol treatment, this value increased to a mean of 165.8 $\pm$ 39.8 mg/g of liver at the end of 21 months. A case of obvious steatosis is shown in Fig. 2. In addition to the fat, mild inflammation, cellular degeneration, and some fibrosis were noted. In three animals fed ethanol for 9 months and one animal fed ethanol for 12 months, alcoholic hepatitis developed, as defined by cell degeneration, inflammation, and central sclerosis (Figs. 2–4).

In the four animals that developed hepatitis, triglyceride accumulation was more pronounced (180.4 $\pm$ 55.7 mg/g) than in the five animals that had only a fatty liver (83.0 $\pm$ 5.7 mg/g). Two of the four animals that had developed hepatitis after 9 months were again subjected to biopsy after

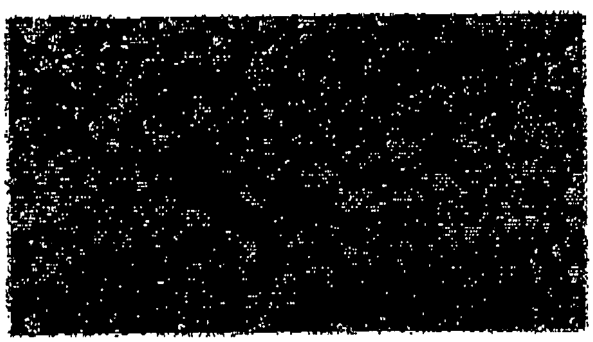

FIG. 2. Section of liver of baboon no. 536 after 9 months of ethanol administration. Severe fatty liver is present. Diffuse interstitial fibrosis and central sclerosis (arrow) are prominent. Chromotrope-aniline blue (magnification: ×44).

FIG. 4. High power view of liver shown in Fig. 3. Area of central necrosis and fibrosis, showing polymorphonuclear leukocytes (arrows). Hematoxylin and eosin (magnification: ×444).

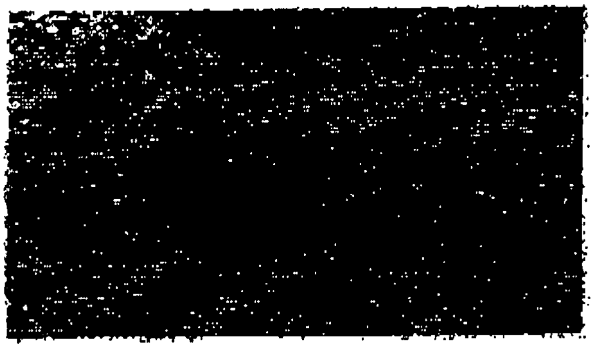

FIG. 5. Section of liver of baboon no. 536 after 20 months of ethanol administration. Connective tissue septa have formed, and in one area have already circumscribed a nodule. This case was classified as incomplete cirrhosis. Chromotrope-aniline blue (magnification: ×44).

20 months. Each showed the development of extensive fibrosis, corresponding to a diagnosis of incomplete cirrhosis. The progress of the lesions from the alcoholic hepatitis to cirrhosis is shown in Fig. 5. One of the animals died after 2 years of treatment because of withdrawal symptoms (convulsions) which developed when the intake of ethanol decreased because of an intercurrent infection. The autopsy revealed typical complete alcoholic Laennec's cirrhosis of the liver (Fig. 6). Another animal that showed alcoholic hepatitis also died after 18 months of treatment because of a similar complication. The autopsy revealed only fatty liver. When alcohol intake was decreased for reasons of intercurrent upper respiratory infection, withdrawal symptoms (such as tremor and seizures) were observed in at least four animals.

Ultrastructural changes were already pronounced in the fatty livers; they remained present throughout the stages of hepatitis and cirrhosis. The mitochondrial lesions of the fatty livers were characterized by enlargement, irregular forms, and disoriented cristae. The rough endoplasmic reticulum was decreased, and the smooth endoplasmic reticulum was vesicular and proliferated (Fig. 7). Similar lesions were found in the livers displaying alcoholic hepatitis and cirrhosis.

There was an increase in the activity of the microsomal ethanol-oxidizing system in the animals fed ethanol: 23.0 ± 2.5 nmol/min per mg of protein compared to 18.7 ± 1.5 in the controls ($P < 0.01$). It is noteworthy, however, that of the four animals that had the hepatitis, only two had a significant increase in the activity of the microsomal ethanol-oxidizing system, whereas the two others had values comparable to that of the corresponding controls. We have reported previously that other microsomal enzymes also increase in activity after ethanol feeding, both in rats (14, 15) and in baboons (10). This was confirmed for microsomal aniline hydroxylase activity measured in 4 pairs of baboons: whereas the mean value in the animals fed ethanol was 0.874 nmol/min per mg of microsomal protein, the corresponding controls had activities of 0.299.

Serum cholesterol was moderately increased in the alcohol-treated baboons (214.1 ± 22.2 compared to 153.6 ± 11.0 mg/100 ml, $P < 0.05$). In all pairs of animals, the values of serum glutamic-oxaloacetic transaminase were higher in the ethanol-fed animals than in the corresponding controls. In the five animals with a simple fatty liver, however, the increase was small. By contrast, in the four animals that had hepatitis, there were striking elevations of 2650, 227, 123, and 75 IU/ml, as compared to values of 40, 37, 35, and 38 in the corresponding controls. No significant abnormalities were noted in albumin, bilirubin, alkaline phosphatase activity, creatinine, urea, and glucose. Hemoglobin and hematocrit values had a tendency to be lower in the alcohol-fed animals, but no meaningful decrease has been noted thus far.

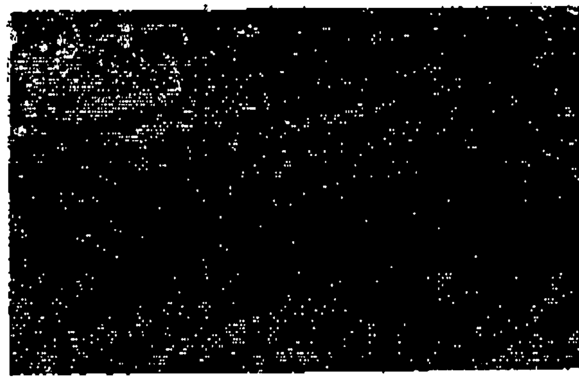

FIG. 6. Complete cirrhosis of liver of baboon no. 536 after 24 months of ethanol administration. Broad connective tissue septa circumscribe nodules containing irregularly arranged hepatocytes and fat. Chromotrope-aniline blue (magnification: ×44).

FIG. 7. Electron micrograph of liver during fatty liver stage. Mitochondria (M) are enlarged and misshapen, and exhibit disoriented cristae. Rough endoplasmic reticulum is sparse, while smooth endoplasmic reticulum (SER) is increased. Fat, F (magnification: ×7992).

An additional group of 12 animals that had been given a solid diet with either alcohol or carbohydrates in the drinking water as described before (10) for a period varying from 17 to 34 months were then changed to the liquid diet for an average of 17 months. Whereas when alcohol was given with the solid diet no lesions more severe than fatty liver had developed (10), with this new regimen, four of the six animals fed alcohol progressed to a more severe stage: one to alcoholic hepatitis (after 29 months of the solid diet and 19 months on the liquid diet), two to incomplete cirrhosis (after 30 months on the solid diet and 15 months on the liquid diet), and one to complete cirrhosis (after 34 months on the solid and 19 months on the liquid diet). Some of these preliminary findings were previously reported (16).

DISCUSSION

The present study establishes the fact that in subhuman primates, chronic ingestion of alcohol can cause the development of the entire spectrum of liver lesions seen in man, namely, fatty liver, alcoholic hepatitis, and cirrhosis. Out of 15 animals fed alcohol, cirrhosis developed in five. In two animals, this complication appeared as early as 2 years after the administration of a totally liquid diet containing 50% of total calories as alcohol; three other animals developed cirrhosis after this regimen was given for 17 months after administration for 30–34 months of alcohol as part of drinking water (10). Fatty liver was observed in all animals given alcohol. Alcoholic hepatitis, characterized by inflammation and central sclerosis (17), was observed in five animals.

The results of this study are significant, both with regard to the understanding of the pathogenesis of alcoholic liver injury and to its treatment. The experimental reproduction of the lesions of alcoholic hepatitis and the demonstration in an experimental model of its transition to cirrhosis support the hypothesis that alcoholic hepatitis is a precursor of the cirrhotic lesion. Moreover, this study shows that animals that displayed fatty liver with a moderate alcohol intake developed hepatitis and cirrhosis when the alcohol content of the diet was increased; this raises the question whether the fatty liver can be considered as a precursor state for the hepatitis and cirrhosis. This possibility is supported not only by the temporal relationship of the fatty liver, which always preceded the development of hepatitis and cirrhosis, but also by the observation that the ultrastructural changes observed at the fatty liver stage are already as pronounced as those seen when a full blown hepatitis or cirrhosis had developed. In addition, it was found that already in the fatty liver phase there was an increase in chemically detectable collagen, the protein which is the hallmark for the fibrosis characteristic of cirrhosis (18): this was associated with enhanced activity of peptidylproline hydroxylase, an enzyme active in the initial steps of fibrogenesis (18).

The present study also clarifies the respective role of malnutrition and alcohol itself in the pathogenesis of the alcoholic hepatitis and cirrhosis. Our previous observations have shown that the fatty liver can be produced by ethanol *per se* in the absence of dietary deficiencies (2–4). We now find that this also applies to the hepatitis and the cirrhosis. It is noteworthy that although fibrosis and cirrhosis have been produced before in primates after the feeding of deficient diets lacking in protein and/or choline (19–21), these animals did not develop hepatitis, a rather characteristic stage in alcoholic liver injury. Furthermore, the striking ultrastructural changes produced by alcohol in man (3, 4, 22), in rats (23), or in baboons in this study differ strikingly from the ultrastructural changes ascribed to choline (20) and/or protein (24) deficiencies. One may, therefore, conclude from our studies that despite the evidence produced before indicating that malnutrition can cause liver damage, alcohol itself is an indispensable etiologic agent for the development of the typical complications observed in the alcoholic. An important corollary of this finding is the fact that adequate diet did not prevent the development of the alcoholic lesions. The therapeutic implication of this observation is that alcoholics cannot fully prevent the development or the aggravation of liver injury by maintaining an adequate diet unless they also control the degree of alcohol intake. It has been shown in the past by others and our own group that alcohol ingestion results in impaired digestion and in malabsorption and that it produces intestinal injury (25–29). It is unlikely, however, that the effects described are of sufficient magnitude to offset the large excess of nutrients present in our diet. Moreover, preliminary studies have indicated the absence of protein and fat malabsorption under our experimental conditions (J. Lindenbaum and C. S. Lieber, unpublished observation). The possibility that nutritional deficiencies may potentiate the effect of alcohol is presently being investigated in the baboon, since such a phenomenon was observed in the rat (30).

We thank Dr. J. Moor-Jankowski, Dr. E. Muchmore, Dr. W. Niemann, and their entire LEMSIP staff for constant advice and help, to Miss N. Lowe and Mrs. E. Jayatilleke for expert technical assistance. This study was supported, in part, by USPHS Grants AA-00224, AM-12511, and the Veterans Administration Project no. 5251-01.

1. City of New York Department of Health (1969) *Mortality from Cirrhosis of the Liver, New York City 1949–1968* (Bureau of Records and Statistics, Statistical Division).
2. Lieber, C. S., Jones, D. P. & DeCarli, L. M. (1965) *J. Clin. Invest.* 44, 1009–1021.
3. Lieber, C. S. & Rubin, E. (1968) *Amer. J. Med.* 44, 200–206.
4. Rubin, E. & Lieber, C. S. (1968) *N. Engl. J. Med.* 278, 869–876.
5. Lieber, C. S. & DeCarli, L. M. (1974) *J. Med. Primatol.* 3, 153–163.
6. *Food and Nutrition Board: Recommended Dietary Allowances* (1973) (National Academy of Sciences, Washington, D.C.), rev. 8th ed.
7. Foy, H., Kondi, A. & Mbaya, A. (1964) *Brit. J. Nutr.* 18, 307–318.
8. Portman, O. W. (1970) in *Feeding and Nutrition of Nonhuman Primates*, ed. Harris, R. S. (Academic Press, New York), pp. 87–115.
9. Hummer, R. L. (1970) in *Feeding and Nutrition of Non human Primates*, ed. Harris, R. S. (Academic Press, New York), pp. 183–203.
10. Lieber, C. S., DeCarli, L. M., Gang, H., Walker, G. & Rubin, E. (1972) in *Medical Primatology-1972*, eds. Goldsmith, E. I. & Moor-Jankowski, J. (S. Karger, Basel), part 3, pp. 270–278.
11. Lieber, C. S. & DeCarli, L. M. (1970) *J. Biol. Chem.* 245, 2505–2512.
12. Imai, Y., Ito, A. & Sato, R. (1966) *J. Biochem.* 60, 417–428.
13. Bonnichsen, R. (1963) in *Methods of Enzymatic Analysis*, ed. Bergmeyer, H. U. (Academic Press, New York), pp. 285–289.
14. Rubin, E. & Lieber, C. S. (1968) *Science* 162, 690–691.
15. Ishii, H., Joly, J. -G. & Lieber, C. S. (1973) *Biochim. Biophys. Acta* 291, 411–420.
16. Rubin, E. & Lieber, C. S. (1974) *N. Engl. J. Med.* 290, 128–135.

17. Galambos, J. T. (1974) in *The Liver and its Diseases*, eds. Schaffner, F., Sherlock, S. & Leevy, C. M. (Intercontinental Medical Book Corp., New York), pp. 255–267.
18. Feinman, L. & Lieber, C. S. (1972) *Science* 176, 795.
19. Hoffbauer, F. W. & Zaki, F. G. (1965) *Arch. Pathol.* 79, 364–369.
20. Ruebner, B. H., Moore, J., Rutherford, R. B., Seligman, A. M. & Zuidema, G. D. (1969) *Exp. Mol. Pathol.* 11, 53–70.
21. Wilgram, G. F. (1959) *Ann. Intern. Med.* 51, 1134–1158.
22. Lane, B. P. & Lieber, C. S. (1966) *Amer. J. Pathol.* 49, 593–603.
23. Iseri, O. A., Lieber, C. S. & Gottlieb, L. S. (1966) *Amer. J. Pathol.* 48, 535–555.
24. Patrick, R. S., MacKay, A. M., Coward, D. G. & Whitehead, R. G. (1973) *Brit. J. Nutr.* 30, 171–179.
25. Lindenbaum, J. & Lieber, C. S. (1969) *Nature* 224, 806.
26. Tomasulo, P. A., Kater, R. M. H. & Iber, F. L. (1968) *Amer. J. Clin. Nutr.* 21, 1340–1344.
27. Halsted, C. H., Robles, E. A. & Mezey, E. (1973) *Gastroenterology* 64, 526–532.
28. Rubin, R., Rybak, B., Lindenbaum, J., Gerson, C. D., Walker, G. & Lieber, C. S. (1972) *Gastroenterology* 63, 801–814.
29. Baraona, E., Pirola, R. C. & Lieber, C. S. (1974) *Gastroenterology* 66, 226–234.
30. Lieber, C. S., Spritz, N. & DeCarli, L. M. (1969) *J. Lipid Res.* 10, 283–287.

Home Help Subjects Feedback Random Search OMD

stillage

The grains and liquid effluent remaining after distillation.

(05 Dec 1998)

Previous: stilbestrol, stilbite, Stiles-Crawford effect, Stiles, Walter, stilet, still
Next: stillbirth, stillbirth rate, stillborn, stillborn infant